

# Osteoarthritis and Cartilage

Journal of the OsteoArthritis Research Society International



## Overexpression of active TGF-beta-1 in the murine knee joint: evidence for synovial-layer-dependent chondro-osteophyte formation

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### Summary

**Objective:** To investigate the impact of a prolonged and constant active TGF- $\beta$  expression by the synovial lining cells on cartilage and ligamentous joint structures *in vivo*.

**Design:** An adenoviral vector (AdTGF- $\beta$ 1<sup>223,225</sup>) was used for the overexpression of active TGF- $\beta$ 1 in knee joints of C57Bl/6 mice.

**Results:** It was found that physiological relevant levels of active TGF- $\beta$ 1 produced by the synovial lining layer resulted in histopathological changes: hyperplasia of synovium and chondro-osteophyte formation at the so-called chondro-synovial junctions. No histological changes were seen after intra-articular injection of an empty control vector (AdDL70-3) or by overexpression of latent TGF- $\beta$ 1 (AdTGF- $\beta$ 1). The predominant site of TGF- $\beta$  production in osteoarthritis (OA) and rheumatoid arthritis (RA) is the synovial lining layer. To address the question whether the TGF- $\beta$ -induced changes were related to the expression site in the synovial lining, the synovial lining layer was depleted by local treatment with liposomes encapsulating clodronate. Depletion of the lining resulted in a dramatic change of TGF- $\beta$ 1-induced pathology: markedly reduced chondro-osteophyte formation and increased accumulation of extracellular matrix in the synovium.

**Conclusion:** This study shows that overexpression of active TGF- $\beta$ 1 in the knee joint results in OA-like changes and suggests the synovial lining cells contribute to the chondro-osteophyte formation. © 2001 OsteoArthritis Research Society International

**Key words:** TGF- $\beta$ , Chondro-osteophyte formation, Cartilage, Adenoviral vector.

### Introduction

Transforming growth factor- $\beta$  (TGF- $\beta$ ) is a pluripotent factor. Its main effects on cells are growth inhibition of hematopoietic, epithelial and endothelial cells, stimulation of chemotaxis of cells including lymphocytes, macrophages and fibroblasts, and stimulation of extracellular matrix formation by mesenchymal cells<sup>1,2</sup>. Most cells constitutively express TGF- $\beta$  mRNA<sup>2</sup>. TGF- $\beta$  is produced in a latent form that does not bind to TGF- $\beta$  receptors. Regulation of TGF- $\beta$  activity occurs by activation of the latent complex, modulation of synthesis by growth factors including TGF- $\beta$ , scavenging of mature TGF- $\beta$  by  $\alpha_2$ -macroglobulin and down-regulation of receptors<sup>3</sup>. High levels of active TGF- $\beta$ 1 have been found in synovial fluids of osteoarthritis (OA) and rheumatoid arthritis (RA) patients<sup>4–9</sup>. TGF- $\beta$  might play a dual role in RA. On the one hand, TGF- $\beta$ 1 is proposed to account for most of the immunosuppressive activity in the synovial fluid of these patients<sup>6</sup>, but on the other hand TGF- $\beta$  is implicated in fibrosis of the synovium<sup>10</sup>.

We previously demonstrated that multiple intraarticular injections using high bolus amounts of acid activated

TGF- $\beta$ 1-induced hyperplasia of synovium and chondro-osteophytes at the chondro-synovial junctions and at ligament insertions<sup>11–13</sup>. In addition, formation of cartilage-like tissue in ligaments and the synovial membrane was observed. Although, with the injection protocol we could not mimic the predominant site of TGF- $\beta$  production as seen in OA and RA, immunohistological studies have shown that the synovial membrane is one of the major sites of TGF- $\beta$ 1 production in human arthropathies<sup>14–18</sup>. Therefore, in this study we used adenovirally mediated overexpression of active TGF- $\beta$ 1 in the joint. Adenoviral vectors have been shown to efficiently transfect synovial cells in rabbit and murine knee joints<sup>19–23</sup>. The adenoviral-induced expression of TGF- $\beta$  was restricted to the synovial lining and mimics the continuous TGF- $\beta$  exposure of joints during arthropathies. Our data underlines the impressive potency of TGF- $\beta$  to induce joint pathology: hyperplasia of the synovium, chondro-osteophyte formation at the sites of the chondro-synovial junctions and enhanced glycosaminoglycan content of articular cartilage. To study whether the cells producing TGF- $\beta$  play a role in the TGF- $\beta$ -induced pathology, we depleted the synovial lining by local treatment with liposomes encapsulating clodronate<sup>24–27</sup>.

In lining-depleted joints, both adenoviral overexpression of active TGF- $\beta$ 1 and multiple injections of recombinant TGF- $\beta$  protein resulted in extensive fibrosis and an impressive inhibition of chondro-osteophyte formation. This implicates that lining cells could have a major role besides production of TGF- $\beta$  in directing TGF- $\beta$ 1-induced pathology.

Received 27 September 1999; accepted 20 May 2000.

This research was supported by The Dutch League against Rheumatism grant number 941.

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## Materials and methods

### RECOMBINANT ADENOVIRUSES AND TRANSFECTION OF SYNOVIOCYTES

Three recombinant adenoviruses were used. Two contained the cDNA of the full length porcine TGF- $\beta$ 1. AdTGF- $\beta$ 1 expressed the wild-type (latent) protein and AdTGF- $\beta$ 1<sup>223,225</sup> contained a mutation of cysteine to serine at positions 223 and 225, resulting in production of active protein<sup>28,29</sup>. AdDL70-3, a recombinant adenovirus lacking an expression cassette<sup>30</sup>, was used as a control vector. Recombinant adenovirus stocks were prepared and characterized using established methods<sup>31</sup>. Recombinant adenovirus stocks, in PBS, 10% glycerol, were diluted in serum free medium and  $10^6$  to  $10^8$  pfu in a total volume of 6  $\mu$ l was intra-articularly injected.

### MULTIPLE INTRA-ARTICULAR INJECTIONS OF TGF- $\beta$ 1

Twenty nanograms or 200 ng recombinant human TGF- $\beta$ 1 (Genentech Inc, San Francisco, CA, U.S.A.) dissolved in 6  $\mu$ l physiological saline, supplemented with 0.1% ultrapure bovine serum albumin (Sigma, St Louis, MO, U.S.A.) was injected into the joint cavity of the right knee three times on alternating days.

### DEPLETION OF MACROPHAGE-LIKE LINING CELLS WITH CLODRONATE-LADEN LIPOSOMES

Multi-lamellar liposomes encapsulating the drug clodronate (dichloromethylene diphosphonate ( $\text{Cl}_2\text{MDP}$ ), were prepared as described previously<sup>32</sup>. Six microliters of a liposome suspension containing 30  $\mu$ g of clodronate was injected into the knee joint space of C57Bl/6 mice. Phagocytic cells ingest the clodronate-laden liposomes, the membrane of the liposomes is disrupted during digestion, the clodronate is released, and subsequently cells die by apoptosis. Intra-articular injection of clodronate-laden liposomes into C57Bl/6 mice produced optimal depletion of synovial lining cells seven days after injection<sup>24</sup>.

### HISTOLOGY

Total knee joints were dissected, fixed in 4% formaldehyde and processed for histology as described previously<sup>33</sup>. Standard frontal paraffin sections (7  $\mu$ m) were prepared and stained with safranin O and counterstained with Fast green. To visualize collagen in the sections, a Sirius red F3BA/ Fast green staining was performed on the paraffin sections. The surface area of chondro-osteophytes was measured using the Qwin image processing and analysis system (Leica Imaging Systems Ltd. Cambridge, U.K.). Four chondro-osteophytes per frontal section of a knee joint were measured. These were located at the chondro-synovial junctions of both the patella and the femoral condyles. Chondro-osteophytes were measured in three sections per knee joint in the middle part of the joint, standardized using the growth plates.

### DETERMINATION OF PATELLAR CARTILAGE GLYCOSAMINOGLYCAN CONTENT

Articular cartilage glycosaminoglycan (GAG) content is reflected by safranin O staining intensity in histological

sections. Staining intensity was measured using the Qwin image processing and analysis system (Leica Imaging Systems Ltd. Cambridge, U.K.). A modified protocol as published by van der Kraan *et al.*<sup>34</sup> was used. Red staining intensity was measured by integral measurement of the noncalcified layer, approximately 30–40  $\mu$ m in width. Measurements were corrected for lacunae. The average GAG content in control joints was set to 100.

### IMMUNOHISTOCHEMISTRY OF TGF- $\beta$ 1

Whole murine knee joints were snap frozen in liquid nitrogen. Sections were attached to adhesive tape<sup>35</sup>, fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 10 min, and either used immediately or air-dried and stored at  $-70^\circ\text{C}$  in an airtight container. Sections were rehydrated in PBS and endogenous peroxidase was blocked by incubation in 0.5%  $\text{H}_2\text{O}_2$  in PBS for 30 min with continuous shaking. Active TGF- $\beta$  was detected as described<sup>36</sup>. Briefly, after two washes in PBS, sections were incubated overnight at  $4^\circ\text{C}$ , with polyclonal chicken anti-TGF- $\beta$  antibodies (AB-101-NA, R&D systems, Minneapolis, MN) diluted 1:500 in 3% BSA in PBS with 1 M of NaCl. Sections were washed four times in PBS and incubated for 4 h at room temperature with peroxidase conjugated rabbit anti-chicken IgY (Jackson Immuno-research, West Grove, PA) diluted 1:500 in PBS, 3% BSA. After four washes in PBS, peroxidase activity was detected by a 10-min incubation in 0.5 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (Sigma, St Louis, MO) dissolved in 0.05 M TrisHCl buffer, pH 7.6, containing 0.02%  $\text{H}_2\text{O}_2$ . Finally, sections were counterstained with Mayer's hematoxylin and embedded in a gelatin-glycerin solution.

### TGF- $\beta$ BIOASSAY

The patella and surrounding tissue was dissected from murine knee joints injected intraarticularly with AdTGF- $\beta$ 1<sup>223,225</sup> and incubated for 1 h in serum free medium (DMEM). Active TGF- $\beta$ 1 in the medium was measured using a bioassay<sup>37</sup>. Briefly,  $6 \times 10^4$  MLEC cells (a kind gift from Dr D. B. Rifkin) were plated per well in a 96-well plate. Cells were allowed to attach for 4 h at  $37^\circ\text{C}$ . Then medium was replaced with 100  $\mu$ l activated standards or samples in DMEM/BSA. Cells were incubated for 20 h at  $37^\circ\text{C}$  in a  $\text{CO}_2$  incubator. All further steps were carried out on ice. Cells were washed with PBS and cell lysates were prepared. Luciferase in the cell lysates was measured with a luminometer.

### TGF- $\beta$ ELISA

The ELISA is specific for active TGF- $\beta$ 1 and TGF- $\beta$ 2. Soluble type II TGF- $\beta$  receptor (3  $\mu$ g/ml) was coated on a 96-well plate in 0.1 M carbonate buffer (pH 9.6) by incubation of 50  $\mu$ l per well overnight at  $4^\circ\text{C}$ . The 96-well plate was washed three times with PBS, 0.05% Tween 20 and blocked with 1% gelatine in phosphate buffer (pH 7.4), for 1 h at  $37^\circ\text{C}$ . 50  $\mu$ l TGF- $\beta$  samples or TGF- $\beta$  standards per well were incubated for 1 h at  $37^\circ\text{C}$ . Chicken anti-TGF- $\beta$ 1 antibody (R&D systems AB-101-NA) 0.2  $\mu$ g/ml in 0.5% gelatine, PBS, 0.05% Tween 20 was incubated for 1 h at  $37^\circ\text{C}$ . The plate was incubated with rabbit anti-chicken IgY, biotiny labeled, in 0.5% gelatine in PBS, 0.05% Tween 20, 50  $\mu$ l per well for 1 h at  $37^\circ\text{C}$ . The plate was incubated

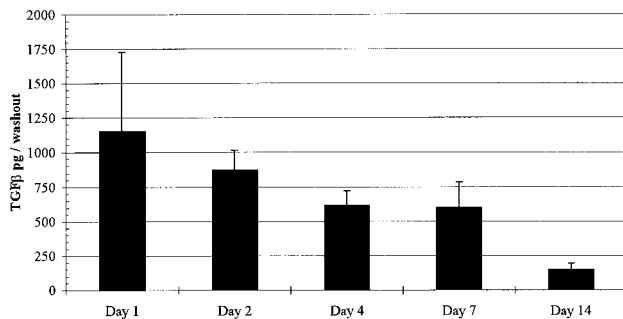


Fig. 1. Intraarticular production of active TGF- $\beta$ 1 at different time points after transfection with  $10^7$  pfu of AdTGF- $\beta$ 1<sup>223,225</sup>, measured with a bioassay in 1 h patella washouts.

with anti-HRPO in PBS, 1% caseine for 30 min at 37°C. Peroxidase activity was detected by incubation with 8 mg per 10 ml o-phenylenediamine in phosphate buffer pH 6.0, with 0.3% H<sub>2</sub>O<sub>2</sub> for 10 min. This reaction was stopped with 50  $\mu$ l 2.5 M H<sub>2</sub>SO<sub>4</sub> per well. Plate was read in an ELISA plate reader at 492 nm.

## Results

### ADENOVIRAL VECTOR MEDIATED INTRA-ARTICULAR PRODUCTION OF ACTIVE TGF- $\beta$ 1

Synovial tissue specimens were taken at different time points after virus injection and incubated for 1 h in culture medium. Active TGF- $\beta$ 1 in the 1-h washouts was measured with a specific bioassay (Fig. 1). One day after injection of  $10^7$  pfu AdTGF- $\beta$ 1<sup>223,225</sup> the synovial washouts contained high amounts of active TGF- $\beta$ 1 ( $1150 \pm 580$  pg/washout). A gradual decline of bioactive TGF- $\beta$ 1 production was observed for the next 7 days to about 50% of the amount at day one. At day 14 after virus injection the joints still produced significant amounts of active TGF- $\beta$ 1 ( $150 \pm 45$  pg/washout). Immunohistochemistry showed a distinct expression of active TGF- $\beta$ 1 in the synovial lining cells throughout the knee joint at 18 h after AdTGF- $\beta$ 1<sup>223,225</sup> injection [Fig. 2(A)]. Three weeks after injection a few positive cells were still found (data not shown). No cells were stained positive for active TGF- $\beta$ 1 in the sublining, endothelium of blood vessels, and in cartilage. No active TGF- $\beta$ 1 was detected with the ELISA or immunohistochemistry in AdDL70-3 injected joints (data not shown).

### TGF- $\beta$ 1 INDUCED HISTOPATHOLOGICAL CHANGES IN THE MURINE KNEE JOINT

Up to  $10^7$  pfu of control AdDL70-3 virus could be injected into the knee joint cavity without inducing an inflammatory response. We injected  $10^7$  pfu or less in the subsequent TGF- $\beta$  studies. Injection of  $10^6$  pfu of AdTGF- $\beta$ 1<sup>223,225</sup> resulted in a rapid but transient influx of inflammatory cells. Eighteen hours after injection, mononuclear cells and a few PMNs were present in the joint cavity, and disappeared within 48 h. The same transient influx of inflammatory cells was seen after a single injection of 100 ng of active TGF- $\beta$ 1 protein in the joint<sup>11</sup>.

At later time points injection of AdTGF- $\beta$ 1<sup>223,225</sup> resulted in marked hyperplasia of the synovium. There was no progression of the hyperplasia between one and three weeks after injection [Fig. 3(A)]. Safranin O staining and

Sirius red staining (data not shown) showed that there was only a small amount of extracellular matrix surrounding the cells [Fig. 3(A)]. Synovial hyperplasia was also apparent 5 days after the last of three intraarticular injections of TGF- $\beta$ 1 protein at alternate days (20 ng or 200 ng) [Fig. 3(D)]. There was an accumulation of fibroblast-like cells along the periarticular side of the collateral ligaments both in the AdTGF- $\beta$ 1<sup>223,225</sup> and TGF- $\beta$ 1 protein injected joints. TGF- $\beta$ 1-induced extracellular matrix production was evident at distinct sites between ligaments and bone in AdTGF- $\beta$ 1<sup>223,225</sup> injected and in TGF- $\beta$  protein injected knee joints [Fig. 3(C)]. These sites showed extensive staining with safranin O, indicating extracellular matrix deposition.

Glycosaminoglycan (GAG) content in patellar cartilage was determined by detecting the safranin O staining in the non-calcified layer with the Qwin image processing and analysis system (Leica Imaging Systems Ltd, Cambridge, U.K.) (Table I). Murine knee joints injected with  $10^6$  pfu of the AdTGF- $\beta$ 1<sup>223,225</sup> virus showed an increase in GAG content compared to normal knee joints of 5.7% at two weeks after injection and 6.3% 3 weeks after injection, but this increase was not significant. Injection of  $10^7$  pfu of the virus did not lead to an increased GAG content 3 weeks after injection. In comparison, triple injections of 20 ng activated recombinant TGF- $\beta$  resulted in a 3.5% increased GAG content and triple injections with 200 ng resulted in a significant increase of 9.8%.

Chondro-osteophyte formation started 1 week after injection of AdTGF- $\beta$ 1<sup>223,225</sup> at the margins of articular cartilage [Fig. 3(A)]. From 1 to 3 weeks after injection the surface area of the chondro-osteophytes in frontal sections increased (Table II). Histology taken 5 days after the last of three TGF- $\beta$ 1 protein (20 ng) injections showed chondro-osteophyte formation at the same location as seen 3 weeks after injection of  $10^6$  pfu AdTGF- $\beta$ 1<sup>223,225</sup> [Fig. 3(C)]. Larger chondro-osteophytes were formed in joints injected with 200 ng of active TGF- $\beta$ 1 protein (Table III). No histopathological changes were found three weeks after injection of control virus AdDL70-3 ( $2 \times 10^7$  pfu) or a vector expressing latent inactive TGF- $\beta$ 1 ( $10^7$  pfu).

### EFFECT OF MACROPHAGE-LIKE SYNOVIAL CELLS ON TGF- $\beta$ 1 INDUCED PATHOLOGY

Lining cells were depleted to investigate the importance of the synovial lining in the different TGF- $\beta$ 1-induced pathological events. Intraarticular injection of clodronate encapsulated in liposomes resulted in complete depletion of the lining cells 7 days later<sup>24</sup>. At this time point joints were injected with  $10^6$  or  $10^7$  pfu of AdTGF- $\beta$ 1<sup>223,225</sup> virus. Two days later, the level of active TGF- $\beta$ 1 in washouts of synovial tissue was the same as in non-depleted joints (Table IV). Immunohistochemistry showed that in the clodronate treated joints, active TGF- $\beta$ 1 expression was restricted to cells resembling fibroblasts now lining the synovial cavity [Fig. 2(B)]. Furthermore, the immunohistochemistry showed that 3 weeks after infection the number of positive cells was the same for the depleted and the nondepleted knee joints (not shown).

Overexpression of TGF- $\beta$ 1 or injection of active TGF- $\beta$ 1 recombinant protein in synovial-lining-depleted joints resulted in a transient influx of inflammatory cells two days after injection, as seen in the normal joints intra-articularly injected with AdTGF- $\beta$ 1<sup>223,225</sup>. One week after injection the clodronate treated joints showed synovial hyperplasia,

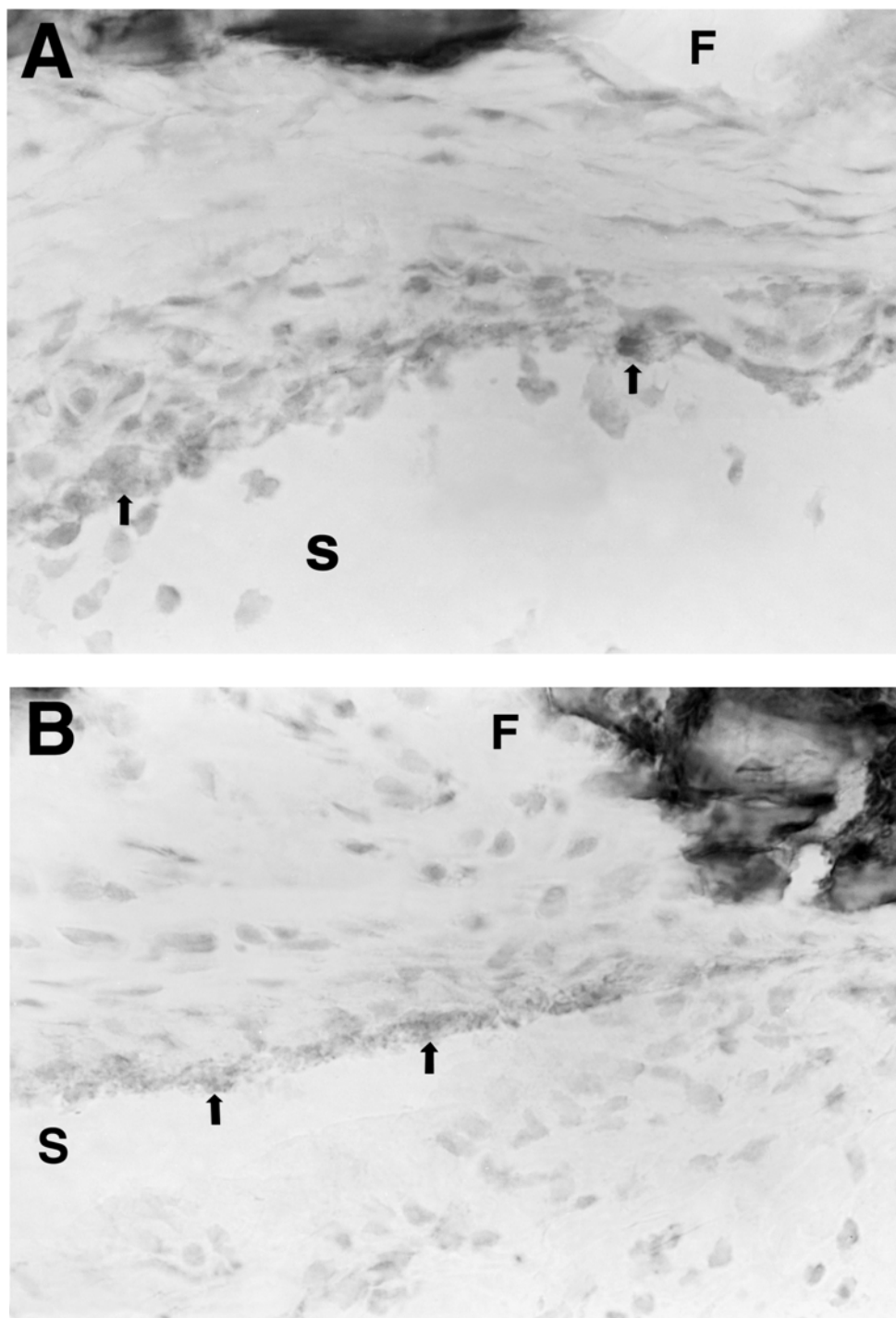


Fig. 2. Immunohistochemistry of active TGF- $\beta$ 1 in cryosections of murine knee joints injected with  $10^7$  pfu of AdTGF- $\beta$ 1<sup>223,225</sup>. Sections were counterstained with heamatoxylin. The arrows indicate the position of synovial lining, which is positive for active TGF- $\beta$ . (A) 18 h after injection in a normal murine knee joint; (B) 18 h after injection in a lining-depleted knee joint. Magnification 250 $\times$ . F, femur; S, joint space.

accompanied by an extensive extracellular matrix deposition, for both the AdTGF- $\beta$ 1<sup>223,225</sup> and the TGF- $\beta$ 1 protein injected joints [Fig. 3(B),(E)].

GAG content in the non-calcified layer of patellar cartilage was determined by detecting the safranin O staining (Table I). Murine knee joints injected with  $10^6$  pfu of the

AdTGF- $\beta$ 1<sup>223,225</sup> virus showed a significant increase of the GAG content of 23.2% at week two and 20.7% at week three after injection. Injection of  $10^7$  pfu resulted in a significant increase in GAG content of 11.9% at 3 weeks after injection. In comparison triple injections of 20 ng active recombinant TGF- $\beta$  protein in clodronate treated



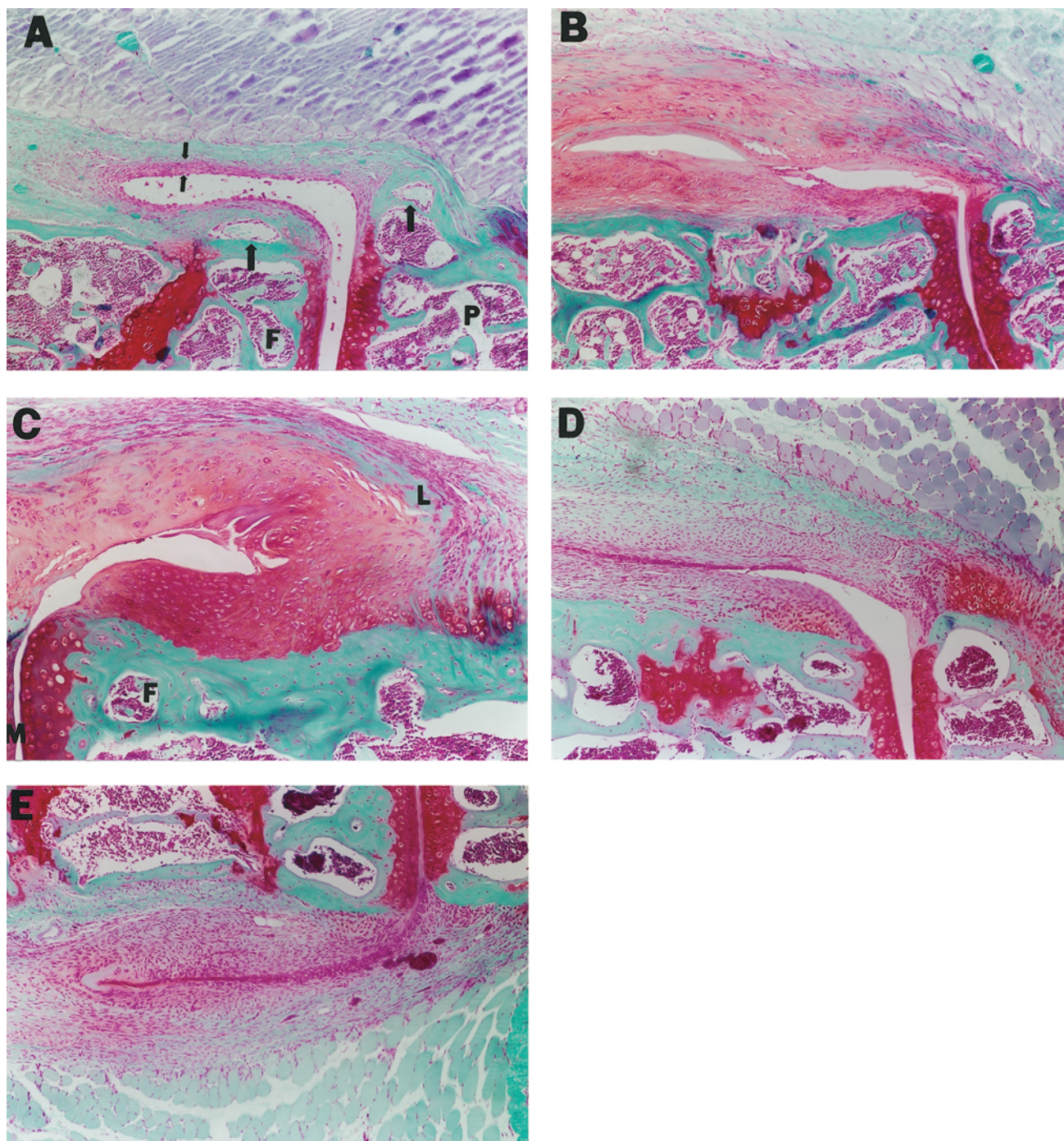


Fig. 3. Safranin O stained paraffin sections of murine knee joints. (A) Three weeks after injection of  $10^6$  pfu AdTGF- $\beta 1^{223,225}$  in a normal knee joint. Large arrows indicate the formation of chondro-osteophytes, small arrows indicate the thickening of the synovial lining layer. (B) Three weeks after injection of  $10^6$  pfu AdTGF- $\beta 1^{223,225}$  in a lining-depleted knee joint. (C) Three weeks after injection of  $10^6$  pfu AdTGF- $\beta 1^{223,225}$  in a normal knee joint. Note the cartilage-like tissue formation between the collateral ligament and bone. (D) Five days after the last of three injections on alternate days with 200 ng TGF- $\beta 1$  in a normal knee joint. (E) Five days after the last of three injections of 200 ng TGF- $\beta 1$  on alternate days in a lining-depleted knee joint. Magnification 100 $\times$  for (A), (B), (D) and (E), and 250 $\times$  for (C). P, patella; F, femur; L, collateral ligament; M, meniscus.

knee joints resulted in an increase of 6.1% 5 days after the last of three injections on alternate days. Triple injections of 200 ng of active recombinant protein resulted in an increase of 13.9% 5 days after the last injection.

In the synovial-lining-depleted joints the TGF- $\beta 1$ -induced chondro-osteophyte formation was impaired [Fig. 3(B),

Tables II and III]. In lining-depleted knee joints exposed to lower amounts of TGF- $\beta$  ( $10^6$  pfu or 20 ng TGF- $\beta$  protein) chondro-osteophyte formation was almost completely absent with only one out of five knee joints having chondro-osteophytes (Table III). In lining-depleted knee joints exposed to higher amounts of TGF- $\beta$  ( $10^7$  pfu

Table I  
Glycosaminoglycan (GAG) content of the patellar cartilage measured by automated image analysis software

Synovial lining	Protein		Adenovirus		
	Day 5		Week 2	Week 3	
	20 ng	200 ng	10 <sup>6</sup> pfu	10 <sup>6</sup> pfu	10 <sup>7</sup> pfu
Normal	103.5±6.6	109.8±4.6*	105.7±3.8	106.3±5.7	98.6±6.0
Depleted	106.1±3.9	113.9±4.9*	123.2±10.1*†	120.7±6.2*†	111.9±3.1†

Knee joints were injected with AdTGF- $\beta^{223,225}$  or three times on alternate days with activated recombinant protein. The relative GAG content was measured as described<sup>34</sup>. Values of the control noninjected knees were set to 100. Values are mean±s.e.

\* $P < 0.05$  compared with the control knees.

† $P < 0.05$  for the clodronate liposome treated knees compared with the non-treated knees, using Student *t*-test.  $N = 5$ .

AdTGF- $\beta^{223,225}$  or 200 ng TGF- $\beta$  protein), the chondro-osteophytes were significantly smaller than in the non-depleted knee joints. This suggests the synovial lining plays a role in the TGF- $\beta$ 1-mediated changes.

## Discussion

High levels of active TGF- $\beta$ 1 have been found in synovial fluids of OA and RA patients<sup>4–9</sup>. In this study we wanted to mimic the relatively constant high concentration of active TGF- $\beta$  in the synovial fluid in arthropathies, using a recombinant adenoviral vector. For the overexpression of TGF- $\beta$  we used the AdTGF- $\beta^{223,225}$  vector<sup>29</sup>. In this vector two point mutations in the TGF- $\beta$  cDNA, changing the Cys codons 223 and 225 in the proregion of the TGF- $\beta$  precursor protein in Ser codons, result in secretion of up to 74% active TGF- $\beta$ <sup>28</sup>. We found that intraarticular AdTGF- $\beta^{223,225}$  injection induced hyperplasia of the synovium and formation of chondro-osteophytes at the chondro-synovial junctions. Furthermore, prior treatment of the knee joint with clodronate encapsulated in liposomes resulted in markedly reduced chondro-osteophytes, and a dramatic increase of extracellular matrix formation around cells in the synovium.

It has been shown that cells lining the joint space can be efficiently transfected *in vivo* by recombinant adenoviruses<sup>19–23</sup>. However, there have been reports of an inflammatory reaction caused by the intra-articular injection of a high amount of adenovirus ( $5 \times 10^8$ )<sup>20</sup>. We found that the adenovirus-induced inflammation, 2 days after injection, was directly related to the injected dosage (data not shown). At a dosage of  $10^7$  pfu, the AdDL70-3 control virus injected joints were histologically hardly distinguishable from vehicle injected joints; there was only a mild widening of the joint cavity, without cellinflux. In subsequent studies we injected  $10^7$  pfu or less in the murine knee joint. When  $10^6$  or  $10^7$  pfu of the AdTGF- $\beta^{223,225}$  was injected a rapid but transient influx of predominantly monocytes was observed. This was probably caused by the presence of active TGF- $\beta$ , because this is a chemoattractant for monocytes<sup>1,2</sup>. Moreover, injection of activated recombinant TGF- $\beta$  showed the same amount of joint inflammation at these early time points.

Histology taken at one, two, and three weeks after intra-articular injection of  $10^6$  or  $10^7$  pfu of the AdTGF- $\beta^{223,225}$  vector showed a progressive hyperplasia in the synovium. There was also some hyperplasia alongside the ligaments and tendons as also seen with the multiple

Table II  
Chondro-osteophyte surface area at the chondro-synovial junctions of the femur and patella of murine knee joints, 1–3 weeks after injection of  $10^6$  pfu AdTGF- $\beta^{223,225}$

Time point	Synovial lining	Surface area in $\mu\text{m}^2$			
		Lateral patella	Medial patella	Lateral femur	Medial femur
1 week	Normal	85±36	76±54	14±16	13±25
	Depleted	0	0	0	0
2 weeks	Normal	218±148	262±178	203±181	116±84
	Depleted	0*	5±11	34±84	19±47
3 weeks	Normal	499±179	593±164	373±198	560±324
	Depleted	0*	30± 73*	18±44*	1±3*

Knee joints were obtained 1, 2 and 3 weeks after injection of  $10^6$  pfu AdTGF- $\beta^{223,225}$ . The surface area of chondro-osteophytes at the chondro-synovial junctions of the patella and the femur [as shown in Fig. 3(A)] were measured using the Qwin image processing and analysis system (Leica Imaging Systems Ltd). Statistics were performed using the Mann-Whitney rank sum test. Lining-depleted knees were compared to the normal knees. Values are mean±s.e.

\* $P < 0.05$ .

$N = 5$ .



Table III  
Chondro-osteophyte surface area at the chondro-synovial junctions of the femur and patella of murine knee joints

Injection	Synovial lining	Surface area in $\mu\text{m}^2$			
		Lateral patella	Medial patella	Lateral femur	Medial femur
$10^6$ pfu	Normal	499 $\pm$ 179	593 $\pm$ 164	373 $\pm$ 198	560 $\pm$ 324
	Depleted	0*	30 $\pm$ 73*	18 $\pm$ 44*	1 $\pm$ 3*
$10^7$ pfu	Normal	727 $\pm$ 472	1264 $\pm$ 674	351 $\pm$ 145	268 $\pm$ 241
	Depleted	0*	357 $\pm$ 265*	237 $\pm$ 218	416 $\pm$ 415
20 ng	Normal	284 $\pm$ 152	342 $\pm$ 211	202 $\pm$ 74	233 $\pm$ 204
	Depleted	0*	0*	18 $\pm$ 40*	20 $\pm$ 32
200 ng	Normal	535 $\pm$ 323	1576 $\pm$ 609	1075 $\pm$ 547	1160 $\pm$ 612
	Depleted	117 $\pm$ 237	186 $\pm$ 276*	205 $\pm$ 210*	466 $\pm$ 417

Knee joints were obtained 3 weeks after injection of the AdTGF- $\beta$ 1<sup>223,225</sup> vector, or 5 days after the last of three intraarticular injections on alternate days with 20 or 200 ng active TGF- $\beta$ 1 protein. The surface area of chondro-osteophytes at the chondro-synovial junctions of the patella and the femur [as shown in Fig. 3(A)] were measured using the Qwin image processing and analysis system (Leica Imaging Systems Ltd). Statistics were performed using the Mann-Whitney rank sum test. Lining-depleted knees were compared with the normal knees. Values are mean $\pm$ S.E.

\* $P < 0.05$ .

N=5.

injection protocol. The TGF- $\beta$ -induced hyperplasia could be the result of its mitogenic effect on synovial cells and interference with the apoptotic process<sup>10</sup>. Furthermore, TGF- $\beta$ 1 is also chemotactic for fibroblasts<sup>1,2</sup>. Between the collateral ligaments and bone a cartilage-like tissue was formed near the attachment site to the femur. This was also seen in the multiple-injection protocol [Fig. 3(C)].

In earlier studies we showed that GAG deposition in patellar cartilage was enhanced as a result of three intra-articular injections of TGF- $\beta$  on alternate days<sup>11,13</sup>. This was confirmed in the present study. In knees injected with  $10^6$  pfu of AdTGF- $\beta$ 1<sup>223,225</sup> the GAG content of the patellar cartilage was enhanced 2 and 3 weeks after injection, but was not significant. However, in knees injected with  $10^7$  pfu of the virus, GAG content was the same as in the control knees. This was unexpected. The reason for this discrepancy between the activated protein-injected knee joints and the virus-injected knee joints could be related to the adenoviral vector. It has been shown that injection of an adenoviral vector into murine lung leads to production of TNF- $\alpha$ , IL-1, and IL-6 from day one until at least day seven<sup>38</sup>. We have shown that TNF- $\alpha$  has an inhibitory effect on articular cartilage GAG synthesis, IL-1 has a very strong inhibitory effect and IL-6 slightly induces GAG synthesis<sup>39</sup>. This indicates that adenoviral vector-induced effects could have a negative influence on GAG content of articular cartilage. IL-1 has been shown to be able to counteract the TGF- $\beta$

effect on GAG synthesis and content<sup>40</sup>; this could result in the 'normal GAG content' of the transfected knee joints.

The lining cells were depleted because immunohistological studies showed that, predominantly, the synovial membrane expressed TGF- $\beta$ 1 in human arthropathies<sup>14-18</sup>. By depletion of the synovial membrane we were able to determine if the cells expressing TGF- $\beta$ 1 are important for the intraarticular TGF- $\beta$ 1 effects. TGF- $\beta$  production 2 days after injection of  $10^6$  or  $10^7$  pfu AdTGF- $\beta$ 1<sup>223,225</sup> in the clodronate-treated knee joints was the same as in normal knee joints. However, dramatic differences were found in TGF- $\beta$ -induced pathology. There was a profound increase in extracellular matrix deposition in the synovium and reduced mobility of the joints. In contrast, chondro-osteophyte formation was impaired. This was not caused by differences in the amount of TGF- $\beta$ 1 expression or an altered expression pattern in the depleted joints at later time points, since the same results were obtained with the repeated injection protocol of activated recombinant TGF- $\beta$ 1 protein. This suggests an important role of the synovial lining cells in directing TGF- $\beta$ 1-induced pathology in the murine knee joint, either by production of TGF- $\beta$ -induced secondary factors, or possibly by virus-related proinflammatory cytokine production by the infected cells. The latter was further examined. Recombinant TGF- $\beta$  protein was injected, which led to the same impaired chondro-osteophyte formation. This indicates that the viral-induced proinflammatory cytokines do not have a qualitative effect on the TGF- $\beta$ -induced chondro-osteophyte formation.

However, at present we cannot exclude that the liposome treatment had an impact on periosteal cells or other phagocytic cell types in the sublining layer of the synovium. Chondro-osteophytes still developed when higher amounts of virus or recombinant protein were injected, indicating that periosteal cells were probably not depleted by the clodronate treatment. Application of macrophage depletion in pathologies in which TGF- $\beta$  plays a role will have major effects on all aspects of the disease, and these changes will influence the TGF- $\beta$  effects. The experiments reported here only show that in the joint, macrophage-like synovial lining cells have a major effect on TGF- $\beta$ -directed joint

Table IV  
Intra-articular production of active TGF- $\beta$ 1 at day two after transfection with AdTGF- $\beta$ 1<sup>223,225</sup>

Synovial lining	Pfu's injected	TGF- $\beta$ (pg/washout)
Normal	$10^6$	241 $\pm$ 40
	$10^7$	438 $\pm$ 14
Depleted	$10^6$	286 $\pm$ 64
	$10^7$	410 $\pm$ 354

Active TGF- $\beta$  was measured in 1-h patella washouts using an ELISA. Values are mean $\pm$ S.E. N=3.

pathology. Currently we are investigating if the synovial lining cells participate in the TGF- $\beta$ 1-induced pathology by releasing secondary cofactors.

In the clodronate-treated knee joints injected with  $10^6$  pfu or  $10^7$  pfu of AdTGF- $\beta^{223,225}$  the GAG content of the patellar cartilage was significantly increased compared with nontreated AdTGF- $\beta^{223,225}$  injected knee joints. GAG content in the knee joints injected three times with recombinant TGF- $\beta$  protein was slightly higher in clodronate-treated knee joints compared to normal knee joints, but was not significant. This again points to the possible involvement of adenovirus-induced inhibitory factors produced by the synovium. It has been described that Kupffer cell depletion, using liposomes encapsulating clodronate, decreases adenoviral-induced proinflammatory cytokine production in the liver<sup>41</sup>. Furthermore, clodronate treatment of knee joints significantly reduces IL-1 production in the collagen-induced arthritis model<sup>27</sup>. This suggests that the increase of GAG content in clodronate treated joints can be caused by a decrease in IL-1 production.

In summary, local production of TGF- $\beta$ 1 in the synovium leads to formation of chondro-osteophytes, synovial hyperplasia, and formation of cartilage-like tissue between the collateral ligaments and bone. Clodronate treatment has no effect on the expression pattern of TGF- $\beta$ , but modulates various intra-articular TGF- $\beta$ 1 effects. It enhances the formation of extracellular matrix in the synovium and it partially inhibits chondro-osteophyte formation.

## Acknowledgments

We thank Dr Purchio (Genentech Inc. San Francisco, California) for the generous gift of recombinant TGF- $\beta$ 1, and Dr N. van Rooijen (Department of Cell Biology and Immunology, Vrije Universiteit, Amsterdam, The Netherlands) for the generous gift of the clodronate liposomes. We thank Onno Arntz, Alwin Scharstuhl and Elly Vitters for technical assistance. This work was supported by a grant from the Dutch League against Rheumatism.

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